Adherent and Invasive *Escherichia coli* Is Associated with Granulomatous Colitis in Boxer Dogs

Kenneth W. Simpson, 1* Belgin Dogan, 1 Mark Rishniw, 2 Richard E. Goldstein, 1 Suzanne Klaessig, 3 Patrick L. McDonough, 3 Alex J. German, 3 Robin M. Yates, 4 David G. Russell, 4 Susan E. Johnson, 6 Douglas E. Berg, 7 Josee Harel, 8 Guillaume Bruant, 8 Sean P. McDonough, 2 and Ynte H. Schukken 3

Departments of Clinical Sciences, 1 Biomedical Sciences, 2 Population and Diagnostic Sciences, 3 and Microbiology and Immunology, 4 College of Veterinary Medicine, Cornell University, Ithaca, New York; the University of Liverpool, Liverpool, United Kingdom 5; the Ohio State University, Columbus, Ohio 6; Washington University, St. Louis, Missouri 7; and the University of Montreal, Montreal, Canada 8

Received 12 January 2006/Returned for modification 16 February 2006/Accepted 5 May 2006

The mucosa-associated microflora is increasingly considered to play a pivotal role in the pathogenesis of inflammatory bowel disease. This study explored the possibility that an abnormal mucosal flora is involved in the etiopathogenesis of granulomatous colitis of Boxer dogs (GCB). Colonic biopsy samples from affected dogs (*n* = 13) and controls (*n* = 38) were examined by fluorescent in situ hybridization (FISH) with a *universal* 16S rRNA probe. Culture, 16S ribosomal DNA sequencing, and histochemistry were used to guide subsequent FISH. GCB-associated *Escherichia coli* isolates were evaluated for their ability to invade and persist in cultured epithelial cells and macrophages as well as for serotype, phylogenetic group, genome size, overall genotype, and presence of virulence genes. Intramucosal gram-negative cocccobacilli were present in 100% of GCB samples but not controls. Invasive bacteria hybridized with FISH probes to *E. coli*. Three of four GCB-associated *E. coli* isolates adhered to, invaded, and replicated within cultured epithelial cells. Invasion triggered a “splash”-type response, was decreased by cytochalasin D, genistein, colchicine, and wortmannin, and paralleled the behavior of the Crohn’s disease-associated strain *E. coli* LF 82. GCB *E. coli* and LF 82 were diverse in serotype and overall genotype but similar in phylogeny (B2 and D), in virulence gene profiles (*fyuA, irp1, irp2, chuA, fepC, ibeA, kpsMIII, iss*), in having a larger genome size than commensal *E. coli*, and in the presence of novel multilocus sequence types. We conclude that GCB is associated with selective intramucosal colonization by *E. coli*. *E. coli* strains associated with GCB and Crohn’s disease have an adherent and invasive phenotype and novel multilocus sequence types and resemble *E. coli* associated with extraintestinal disease in phylogeny and virulence gene profile.

There is mounting evidence that inflammatory bowel disease (IBD) is a consequence of an overly aggressive immune response to luminal commensal bacteria in genetically susceptible individuals (63, 70). Mechanistic studies of the interplay between the intestinal mucosa and bacteria in rodents with engineered susceptibility, e.g., interleukin 10-negative (IL-10−/−) and IL-2−/− mice and HLA-B27, 13) and controls (*n* = 38) were examined by fluorescent in situ hybridization (FISH) with a *universal* 16S rRNA probe. Culture, 16S ribosomal DNA sequencing, and histochemistry were used to guide subsequent FISH. GCB-associated *Escherichia coli* isolates were evaluated for their ability to invade and persist in cultured epithelial cells and macrophages as well as for serotype, phylogenetic group, genome size, overall genotype, and presence of virulence genes. Intramucosal gram-negative cocccobacilli were present in 100% of GCB samples but not controls. Invasive bacteria hybridized with FISH probes to *E. coli*. Three of four GCB-associated *E. coli* isolates adhered to, invaded, and replicated within cultured epithelial cells. Invasion triggered a “splash”-type response, was decreased by cytochalasin D, genistein, colchicine, and wortmannin, and paralleled the behavior of the Crohn’s disease-associated strain *E. coli* LF 82. GCB *E. coli* and LF 82 were diverse in serotype and overall genotype but similar in phylogeny (B2 and D), in virulence gene profiles (*fyuA, irp1, irp2, chuA, fepC, ibeA, kpsMIII, iss*), in having a larger genome size than commensal *E. coli*, and in the presence of novel multilocus sequence types. We conclude that GCB is associated with selective intramucosal colonization by *E. coli*. *E. coli* strains associated with GCB and Crohn’s disease have an adherent and invasive phenotype and novel multilocus sequence types and resemble *E. coli* associated with extraintestinal disease in phylogeny and virulence gene profile.

---

* Corresponding author. Mailing address: VMC2001, Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853. Phone: (607) 253-3251. Fax: (607) 253-3497. E-mail: kws5@cornell.edu.
bacteria, including *Mycobacterium avium* (20, 25, 77, 80). Immunopathological studies describe an increase in immunoglobulin G3 (IgG3) and IgG4 plasma cells, CD4 T cells, and L1- and major histocompatibility complex II-positive cells (25). Several studies have described bacteria within the mucosa of affected dogs, but known enteropathogens such as *Salmonella*, *Campylobacter Yersinia*, and *Shigella* have not been isolated (28, 32, 78, 80). Ultrastructural studies suggest active phagocytosis of bacteria that in some instances resemble *Chlamydia* (78). An attempt to reproduce colitis in Boxer dogs with *Mycoplasma* isolated from the colon and regional lymph nodes of four affected dogs was unsuccessful (6). The predilection for Boxer dogs, with only sporadic cases of this type of colitis reported in non-Boxer dogs, and the absence of a causal infectious agent have led to GCB being considered a breed-specific, immune-mediated disease of unknown etiology (25, 75). However, a favorable outcome has been described in dogs receiving antibiotics such as chloramphenicol (80), and recent reports describe clinical responses to antibiotic regimens containing fluoroquinolones (19, 32) that have also been effective in some patients with Crohn’s colitis and ileocolitis (10, 70). Thus, GCB represents a spontaneous idiopathic inflammatory bowel disease with features in common with ulcerative colitis (macroscopic appearance, regional distribution, immunopathology), Crohn’s disease (granulomatous inflammation, bacteria within macrophages, response to fluoroquinolones) and Whipple’s disease (PAS-positive macrophages, bacteria within macrophages), but it is not identical to any one of these diseases (20, 25, 77, 80).

The present study directly explored the possibility that an uncharacterized infectious agent such as *Tropheryma whipplei* (20, 66) or an abnormal mucosa-associated flora is involved in the etiopathogenesis of granulomatous colitis of Boxer dogs (GCB). A combination of 16S rDNA sequencing and fluorescence in situ hybridization revealed selective intramucosal colonization of GCB biopsy samples by *Escherichia coli*. *E. coli* isolated from the colonic mucosa of affected dogs adhered to, invaded, and persisted in cultured epithelial cells to the same degree as the Crohn’s disease-associated strain *E. coli* LF 82. Invasion of cultured epithelial cells by GCB isolates and LF 82 is consistent with triggered endocytosis and involves the host cytoskeleton and signaling pathways. We determined that GCB-associated *E. coli* and LF 82 are more similar in phylogeny and virulence gene profiles to extraintestinal pathogenic *E. coli* than diarrheagenic *E. coli*. Our findings support the thesis that IBD is a consequence of mucosal colonization by a restricted subset of the luminal microflora in a susceptible individual and point to the association of *E. coli* that resembles extraintestinal pathogenic strains in genotype with chronic intestinal inflammation.

**MATERIALS AND METHODS**

**Patients, controls, and histopathology.** Formalin-fixed paraffin-embedded colonic biopsy samples from 13 Boxer dogs with a diagnosis of granulomatous colitis (age, 1 to 5 years [median, 1.5 years]; 10 females, 3 males) and control tissues from 27 dogs with other forms of colitis (non-GCB; 7 months to 13 years [median, 5 years]; 10 females, 17 males; 5 Boxers, 4 mongrels, 2 German Shepherd Dogs, 2 Border Collies, 2 Doberman Pinschers, and 12 other breeds), and 11 dogs without colonic inflammation (6 months to 11 years [median, approximately 5 years]; 3 females, 8 males; 5 mongrels, 2 German Shepherd Dogs, and 4 other breeds) were sectioned (5 μm), stained with hematoxylin and eosin (H&E) and PAS, and examined by a pathologist (S.P.M.) who was blinded to the origin of the section.

All GCB sections were characterized by the loss of colonic glands, mucosal erosion and ulceration, and the presence of PAS+ macrophages (Fig. 1). In the non-GCB group, colitis was predominantly lymphoplasmacytic (26/27 dogs: 1 Irish Setter had eosinophilic colitis), and nonulcerative (24/27 dogs), with PAS+ macrophages absent or rarely detected. The severity of colitis in the non-GCB group, determined by the presence of ulcers or erosions, architectural changes such as glandular atrophy or dysplasia, alterations in goblet cells, and increases in cellularity, ranged from severe ($n = 2$) through moderate ($n = 13$) to mild ($n = 12$) (67). Aphthous ulcers were observed in two dogs with mild colitis (one rough Collie, one German Shepherd Dog). Mucosal ulceration was only observed in biopsy samples of a 13-year-old Cocker Spaniel with severe colitis.

Fresh colonic tissue was obtained from an additional two Boxer dogs (12- to 13-month-old spayed females with a 9- to 10-month history of bloody diarrhea and tenesmus) during diagnostic endoscopy. Fecal analysis was negative for enteropathogens, *Giardia*, *Campylobacter*, *Salmonella*, *Yersinia*, and *Shigella*. The diarrhea was unresponsive to dietary modification and metronidazole in both dogs. Additional treatment with sulfasalazine and tyllosin in one dog and enrofloxacin in the other produced no response. Both dogs had mild microcytic anemia (hematocrit, 36 and 37%; normal, 40 to 48%) and a mean corpuscular volume of 57 and 61 fl (normal, 63 to 73 fl), and one dog had a low serum iron and iron saturation suggestive of iron deficiency anemia. Abdominal ultrasound revealed mesenteric lymphadenopathy in one dog and a thickened colon in the other. Colonoscopy (Fig. 1A) showed irregular thickened ulcerated mucosa in both dogs. Colonic biopsy samples were collected into sterile tubes for microbial culture and DNA extraction and into formal saline for histopathology and in situ hybridization. Histopathological findings in both dogs were consistent with GCB.

One dog showed a dramatic clinical response to enrofloxacin (9 mg/kg of body weight orally [p.o.] once daily [SID] for 30 days and then 6 mg/kg p.o. SID for 14 days) and tyllosin (5 mg/kg p.o. SID for 7 days), with resolution of diarrhea and clinicopathological abnormalities and with an increase in body weight (+2 kg). A follow-up colonoscopy was performed 5 months after the initial procedure, 3 months after the end of antibiotic treatment.

**FISH and immunohistochemistry.** Formalin-fixed paraffin-embedded histological sections (4 μm) (15 GCB, 27 non-GCB colitis, and 11 normal samples) were mounted on Probe-On Plus slides (Fisher Scientific, Pittsburgh, Pa.) and evaluated by FISH with a eubacterial probe (EUB-383; GCTGCCCTCCGGTAG GAGT) as previously described (65). Briefly, paraffin-embedded biopsy specimens were deparaffinized by passage through xylene (three times, 20 min each), 100% alcohol (20 min), 95% ethanol (20 min) and finally 70% ethanol (20 min). The slides were air-dried. FISH probes 5′ labeled with Cy3 or Cy5 (Integrated DNA Technologies, Coralville, IA) were 5′ end-labeled with Cy3 or Cy5 and hybridized in a hybridization chamber at 46°C for 4 h. The slides were washed in buffer hybridization buffer (20 nM Tris-HCl, 0.1% sodium dodecyl sulfate [SDS], 0.9% NaCl [pH 7.2]). The sections were allowed to hybridize with 30 μl of DNA probe mix in a hybridization chamber at 46°C for 4 h. Slides were washed in wash buffer (hybridization buffer without SDS) at 48°C for 30 min. Hybridized samples were washed in phosphate-buffered saline (PBS), allowed to air dry, and mounted with a ProLong antifade kit (Molecular Probes Inc., Eugene, OR). Sections were examined on an Axioscope 2 (Carl Zeiss Inc., Thornwood, NY) or a BX51 (Olympus America, Melville, NY) epifluorescence microscope, and images were captured with a Zeiss Axiosc or Olympus DP-7 camera, respectively.

Slides spotted with suspensions of cultured *E. coli* DH5α, *Shigella sonnei* (ATCC 25931), *Salmonella enterica* serovar Typhimurium (ATCC 14028), and

Vol. 74, 2006

E. coli and GRANULOMATOUS COLITIS

4779

E. coli and GRANULOMATOUS COLITIS...
clinical isolates of *Yersinia enterocolitica*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Bacteroides fragilis*, *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Streptococcus equi*, *Streptococcus bovis*, *Clostridium perfringens*, *Clostridium difficile*, *Lactobacillus plantarum*, *Listeria monocytogenes*, and *Helicobacter pylori* were used to control probe specificity. Probe specificity was additionally evaluated using tissue sections and bacteria treated with RNase and using the irrelevant probe non-EUB-338 (ACTCCTACGGGAGGCAGC).

Coloniec sections with evidence of bacterial invasion on eubacterial FISH were subsequently evaluated by Gram, Ziehl-Nielsen, and Steiner stains and FISH probes directed against a subset of the *Enterobacteriaceae* (*E. coli*, *Shigella*, *Salmonella*, and *Klebsiella*; *E. coli* 1531 23S rRNA, CATGAATCACAAAGTG GTAAAGGCC) (64) and *E. coli/Shigella* (*E. coli* 16S rRNA, GCAAAAGGTAT TAACCTTTACTCC) (34).

To aid localization of bacteria within the mucosa, representative sections were stained with a monoclonal antibody to vimentin (clone-V9 [Sigma]; 1:50 with 2.5% bovine serum albumin [BSA] in PBS, 1 h) after the fluorescent in situ hybridization procedure. Incubation with the primary antibody was followed by washing (three times, 10 min each, in PBS), incubation with secondary antibodies (chicken anti-mouse immunoglobulin, Alexafluor488 [Molecular Probes]; 1:50 in 2.5% BSA in PBS for 1 h), washing (three times, 10 min each, in PBS), and mounting with antifade.

**Construction of 16S rRNA libraries from GCB mucosa.** DNA was extracted from mucosal biopsy samples of two Boxer dogs with GC and from one of these dogs after remission induced by enrofloxacin (see patient details above) using a QIAamp DNA minikit (QIAGEN Inc., Valencia, CA) according to the manufacturer’s instructions. A 320-bp fragment of the bacterial 16S gene was amplified from 5 μl of this extract using the primers 16SFa (5′ GCTCAGATT GAACGCTGG), 16S fb (5′ TACTGCTGCCTCCCGTA) (30). Cycling parameters were 94°C for 3 min followed by 26 cycles of 94°C for 30s, 63°C for 1 min, and 72°C for 1 min. A final extension was carried out at 72°C for 5 min. To remove potential contaminating DNA, sterile distilled PCR water was filtered through Micron YM-100 filters (Millipore, Bedford, MA) and exposed to UV irradiation for 10 min. Gel-purified PCR amplicons were extracted using a Perfectprep gel cleanup kit (Eppendorf, Westbury, NY) according to the manufacturer’s instructions. Representative 16S rDNA libraries were established on the basis of the 16S rDNA fragments amplified from nucleic acid extracts. PCR products were cloned into a TA cloning vector (pGEM-T Easy; Promega Corp., Madison, WI) according to the manufacturer’s instructions. Candidate clones were screened by restriction digestion, with clones containing the correct-sized fragments further screened by PCR. Clones with positive PCR results were sequenced at the Cornell University BioResource Center using M13 primers and an ABI 3700 automated DNA sequencer and ABI Prism BigDye terminator sequencing kits with AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). DNA sequences obtained with both forward and reverse primers were proofread and then assembled in Editseq (DNAStar, Madison, WI). Partial 16S rDNA sequences were compared to the sequence databases at NCBI (BLAST-n) and the Ribosomal Database Project (RDPII: http://rdp.cme.msu.edu/), with representative sequences of high homology imported into our database. Sequences were aligned using the Clustal-W algorithm in MegAlign (DNAStar, Madison, WI), and data for each biopsy were summarized as a phylogenetic tree.

**Isolation of mucosa-associated bacteria.** Colonic biopsy samples from 2 Boxer dogs with GC were ground in sterile saline with a sterile pestle. Half of the homogenate was incubated in GN broth (BBL, Becton Dickinson, Franklin Lakes, NJ) at 35°C overnight and then subcultured onto Levine EMB (BBL), while the remainder was directly plated onto Trypticase soy agar (5% sheep blood) (BBL), Levine EMB agar (BBL), and Columbia CNA agar (5% sheep blood) (BBL). All plates were incubated at 35°C in 6% CO2 for 18 to 24 h. Suspect bacterial colonies were then identified using a Sensititre system (Trek, Westlake, OH). Bacterial isolates were stored in glycerol broth at −70°C. DNA was extracted from all isolates, PCR amplified with 16S primers, cloned, and sequenced, with sequences incorporated into the phylogenetic analysis of each biopsy.

**FIG. 1.** Clinical and pathological features of granulomatous colitis of Boxer dogs. A. Colonoscopy shows a diffusely thickened and ulcerated mucosa (arrow). B. Histologically, there is severe loss of glandular structure and cellular infiltration (H&E; magnification, ×200). C. Mucosal infiltration with PAS-staining foamy macrophages (inset) is a dominant feature (magnification, ×200).
Evaluation of adhesion and invasion by GCB-associated *E. coli* in cultured cells. (i) Bacterial strains and culture. *E. coli* strains isolated from GCB mucosa were evaluated for their ability to adhere to, invade, and persist within cultured epithelial cells. *E. coli* strain LF 82, isolated from a chronic ileal lesion of a patient with CD (kindly provided by A. Darfeuille-Michaud) (17), and *Salmo-nella enterica* serovar Typhimurium (ATCC 14028) were used as positive controls. *E. coli* DH5α, a nonpathogenic strain, was used as a negative control. Bacterial isolates were stored at −80°C, and fresh nonpassaged bacteria were used for all investigations. *E. coli* and *S. enterica* serovar Typhimurium were streaked on Luria-Bertani (LB) agar, and a single colony was inoculated into LB broth. Cells were grown overnight at 37°C without shaking. Type 1 pilus expression was confirmed by mannose-sensitive agglutination of 1% commercial baker’s yeast (*Saccharomyces cerevisiae*) suspended in phosphate-buffered saline (PBS) (5, 16). Preliminary studies in our laboratory with strain LF 82 and *Salmonella enterica* serovar Typhimurium confirmed adhesion and invasion of cultured Caco-2 (kindly provided by Andrea Quarioni) and Hep2 (ATCC CCL-25) cells. Strain LF 82 and *Salmonella enterica* serovar Typhimurium also displayed adherent and invasive behavior with cells of the bovine mammary epithelial cell line MAC-T (Nexia Biotechnologies Inc., Montreal, QC, Canada). Monolayers of all cell lines were kept at 37°C in 5% CO₂−95% air (vol/vol) using Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Gemini Bio Products, Woodland, CA) for MAC-T and Caco-2 cells. Hep2 cells were grown in RPMI 1640 (Grand Island, NY) supplemented with 10% FBS. The FBS concentration was dropped to 5% before infection assays.

(ii) Cell culture. Stationary-phase bacteria were pelleted, washed with excess phosphate-buffered saline (PBS, pH 7.4), and resuspended in PBS. Initial bacterial numbers were determined simultaneously by plate count. Confluent monolayers of cells grown in 24-well plates (3 × 10⁴ cells/well) were washed twice with 1 ml/well of growth medium, and then 1 ml of fresh medium was added to each well. Epithelial cells infected with a multiplicity of infection (MOI) of 10 bacteria per epithelial cell. After 1 h of incubation at 37°C with 5% CO₂, cells were washed three times in PBS and lysed in 0.1% Triton X-100 in PBS for 10 min. Lysates were serially diluted and plated on LB agar plates, and colonies were enumerated following overnight incubation. The levels of adhesion were determined throughout the 1-h invasion period. After the 1-h infection and a 2-h incubation, respectively. DMSO (0.1% final concentration) was used as a control. The viability of epithelial cells before and after infection was assessed by immunostaining of extra- and intracellular bacteria. The MIC of gentamicin for all strains was 0.5 μg/ml. Gentamicin killing period, the numbers of intracellular bacteria were determined as described above. Results are reported as the percentage of the number of bacteria that were internalized in control cells with no inhibitor. The viability of epithelial cells was determined in uninfected and infected epithelial cells in the presence and absence of inhibitors.

(v) Differential immunostaining of extra- and intracellular *E. coli*. MAC-T cells seeded and grown overnight on glass eight-well chamber slides were infected with *E. coli* at an MOI of 100. After 3 h of incubation, the cells were washed three times in PBS and fixed with 3.7% formaldehyde for 15 min. PBS containing 10% fetal bovine serum (FBS) was used as blocking buffer and for dilution of immunoreagents. Polyclonal rabbit anti-*E. coli* antibody (B65001R [Biodesign, Saco, ME]; 1:50 in PBS with 10% FBS) was used as the primary antibody. All incubations were carried out at room temperature. For differential immunostaining of extra- and intracellular *E. coli*, the fixed cells were incubated with blocking buffer for 15 min to reduce nonspecific binding of antibodies. Thereafter, the monolayer was incubated with primary antibody for 1 h at room temperature. After washing three times for 10 min each in PBS, monolayers were incubated in the dark with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibodies (F0382 [Sigma-Aldrich, St. Louis, MO]; 1:100 in PBS with 10% FBS) for 45 min. Unbound secondary antibody was removed by washing three times for 10 min each in PBS. Cells were permeabilized with PBS containing 0.1% Triton X-100 for 15 min. After permeabilization, the monolayers were incubated with blocking buffer for 15 min and then with primary antibody for 1 h. After washing three times for 10 min each in PBS, treated monolayers were incubated in the dark with tetramethyl rhodamine isothiocya-nate (TRITC)-conjugated goat anti-rabbit IgG antibodies (T677 [Sigma-Al-drich]; 1:100 in PBS with 10% FBS) for 45 min. Unbound secondary antibody was removed by washing three times for 10 min each in PBS. Slides were mounted with ProLong antifade (Molecular Probes Inc., Eugene, OR) mixed with DAPI (4',6'-diamidino-2-phenylindole) and examined with an Olympus BX51 epifluorescence microscope.

(vi) Transmission electron microscopy. MAC-T cells grown on 24-mm collagen Transwell inserts (Costar, Corning Inc., Corning, NY) and infected at an MOI of 100 were maintained in culture for 3 h. Culture supernatants were removed, and cells were washed twice with 1 ml/well times in culture media before being fixed in 2% glutaraldehyde in PBS, postfixed in 1% osmium tetroxide, dehydrated through ethanol, and embedded in Spurr’s resin. Thin sections were cut, stained with uranyl acetate and Reynolds’ lead, and examined in a Tecnai electron microscope.

Persistence within epithelial cells and macrophages. (i) Epithelial cells. The invasion assay was modified by incubating infected monolayers up to 68 h. After the 1-h invasion and 2-h incubation with 100 μg/ml of gentamicin, cells were washed once in PBS, and fresh medium containing 15 μg of gentamicin/ml was added to the cells. This concentration of gentamicin was used to prevent extra-cellular bacterial growth while reducing the chances of gentamicin leaching into the host cells during longer incubation times. Numbers of intracellular bacteria were determined at 2, 24, and 48 h as described above.

When infected with macrophages. Macrophages derived from the bone marrow of C57BL/6 mice were maintained in Dulbecco’s modified Eagle’s medium (DME-MEM) supplemented with 10% FCS, 5% horse serum, 2 mM l-glutamine, 1 mM sodium pyruvate, and 20% L-cell conditioned media. Fully differentiated macrophages were transferred to coverslips in 24-well plates and incubated for 12 h to allow a confluent monolayer to establish (cell density of 4.2 × 10⁵, in 1 ml of medium). Before infection, the cell monolayers were washed twice with PBS, and the medium was replaced with 1 ml of infection media. Macrophages were infected at a multiplicity of infection (MOI) of 10 bacteria per macrophage, and the plates were centrifuged at 500 × g for 5 min. After a 30-min incubation at 37°C with 5% CO₂, infected macrophages were washed twice with PBS, and fresh cell medium containing 100 μg/gentamicin/ml was added to kill extracellular bacteria. After incubation for an additional 2 h, the medium was removed and fresh medium containing 15 μg of gentamicin/ml was added for longer postinfection periods. To measure intracellular survival beyond 48 h postinfection, fresh cell culture medium containing gentamicin (15 μg/ml) was added to the infected cells. At specified time points, coverslips were washed three times with PBS, and cells were lysed in 2 ml of 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Samples were removed, diluted, and plated onto LB agar plates to determine the number of CFU/well. The number of bacteria surviving gentamicin was determined at 2, 4, 8, 24, 48, and 72 h. Survival was expressed as CFU per well, and the mean percentage of the number of CFU/well determined at 2, 4, 8, 24, 48, and 72 h. The mean number of bacteria surviving gentamicin after 2 h postinfection was defined as 100%. Each experiment was performed in triplicate and repeated at least three times.

Molecular characterization of GCB-associated *E. coli*. The genetic diversity of *E. coli* isolated from GCB mucosa and the Crohn’s disease isolate LF82 was
Microarray experiments were performed in the laboratory of José Harel at the Bio-Rad Laboratories, Hercules, CA, in 0.5 with the restriction enzyme I-PFG. Genomic DNA for PFGE was prepared in agarose plugs and cleaved with the disease isolate LF 82 were determined by pulsed-field gel electrophoresis (one-way ANOVA), where the strain type was used as the 'treatment' variable. Invasion assays with equal variance were analyzed by one-way analysis of variance (ANOVA). Data from adhesion, invasion, and persistence were analyzed using the statistical program Statistix (Analytical Software, Tallahassee, FL).

RESULTS

Granulomatous colitis of Boxer dogs is characterized by a mucosally invasive microflora. The EUB-338 FISH probe hybridized to all reference strains of bacteria. Sections of histologically normal colon had a readily staining bacterial flora that was restricted to the surface mucus layer and colonic glands (Fig. 2A). Bacteria were not detected within the mucosa. Control slides spotted with bacteria and histological sections treated with RNase and the probe non-EUB-338 were negative (data not shown).

In contrast to normal colonic tissue, bacteria in GCB samples were scattered throughout the upper third of the mucosa (Fig. 2B). Clumps of small cocccobacilli (1 to 3 μm) were most commonly observed in areas where goblet cells and glands were replaced by a cellular infiltrate. In many sections bacteria appeared to be localized within cells (Fig. 2C). Histochemical analysis of GCB sections revealed intramucosal gram-negative, non-acid-fast, argyrophilic cocccobacilli, mainly within histiocytes (Fig. 2D), concurring with mucosal localization by FISH. Invasive bacteria were not visible with eubacterial FISH or Gram staining in biopsy samples taken from a GCB-affected dog 3 months after the completion of antibiotic therapy.

To determine if bacterial colonization is a general feature of canine colitis, we examined sections from 27 dogs with non-GCB colitis. Bacterial invasion of the mucosa was observed in only two non-GCB samples, and in these sections the flora was pleomorphic, mixed gram positive and negative, and not cell associated.

Culture and 16S sequencing of GCB mucosa yields predominately Enterobacteriaceae. Culture of GCB mucosa from two affected dogs yielded Klebsiella pneumoniae, Proteus mirabilis, and E. coli. Two E. coli strains were isolated from each dog and were identified as KD-1, KD-2, KD-3 and KD-4. Streptococcus faecalis and Chryseobacterium meningosepticum were isolated from separate individuals. Shigella, Salmonella, Campylobacter, and Yersinia were not isolated.

PCR amplification of DNA from GCB mucosa with 16S primers yielded amplicons of the expected size (320 bp) from pre- and posttreatment samples. Analysis of bacterial sequences yielded a predominance of Enterobacteriaceae, particularly E. coli/Shigella, in both affected dogs (Fig. 3). The posttreatment 16S rDNA library, 3 months after antibiotic-induced remission, contained a much more diverse flora, with only 4/34 clones belonging to Enterobacteriaceae (Fig. 3).

Fluorescence in situ hybridization identifies the intramucosal flora as E. coli or Shigella. On the basis of the results of culture, 16S sequencing, and presence of intramucosal gram-negative bacteria, we employed FISH probes designed to hybridize to a subset of the Enterobacteriaceae (E. coli 1531, 23S rRNA) and E. coli/Shigella (16S rRNA). The specificity of these probes was confirmed with cultured bacteria, with the E. coli 1531 probe hybridizing to E. coli, Shigella, Klebsiella and Salmonella, and with the E. coli/Shigella probe to only these species. Both probes hybridized with the invasive flora of GCB.
specimens (Fig. 4). Culture of feces and mucosa for *Shigella* and PCR of colonic mucosal DNA with primers against the *ipaH* gene found in *Shigella* and enteroinvasive *E. coli* were negative.

GCB-associated *E. coli* adheres to and invades cultured epithelial cells. Four *E. coli* strains (KD1 to -4) were isolated from GCB mucosa from two affected dogs. All of these strains adhered to cultured epithelial cells (Fig. 5A). Strains KD2 to -4 displayed levels of adherence similar to those of the Crohn’s disease isolate LF 82. KD-1, DH5α, and *Salmonella* were less adherent than LF 82 (*P* < 0.05).

In gentamicin protection assays, GCB-associated *E. coli* strains KD1 to -4 and LF-82 were significantly more invasive than DH5α but less invasive than *Salmonella enterica* serovar Typhimurium (*P* < 0.05) on both Caco-2 and MAC-T cells (Fig. 5B and C). GCB strains differed in their invasiveness: strain KD-2 invaded Caco-2 and MAC-T cells to a greater extent than KD-3 and KD-4 and similarly to LF-82. All *E. coli*
strains were more invasive on Caco-2 than MAC-T cells, but the differences between strains were greater on MAC-T than Caco-2 cells. For example, KD-2 invaded Caco-2 cells 25 times more than DH5\textsubscript{A} and MAC-T cells 100 times more than DH5\textsubscript{A}.

The invasive behavior of GCB-associated \textit{E. coli} was independently confirmed using differential immunostaining of extra- and intracellular bacteria and transmission electron microscopy (Fig. 6). Electron microscopy also revealed that GCB-associated \textit{E. coli} induces alterations in the cell membrane characterized by elongations and actin condensation that are consistent with induced endocytosis by a “trigger” process (14). Internalized \textit{E. coli} appeared to be in a close vacuole, but its exact location within the endosomal lysosomal continuum remains to be determined.

Invasion by GCB-associated \textit{E. coli} is dependent on an intact host cell cytoskeleton and signaling pathways. The invasion of epithelial cells by GCB-associated \textit{E. coli} induces alterations in the cell membrane characterized by elongations and actin condensation that are consistent with induced endocytosis by a “trigger” process (14). Internalized \textit{E. coli} appeared to be in a close vacuole, but its exact location within the endosomal lysosomal continuum remains to be determined.

\textbf{Invasion by GCB-associated \textit{E. coli} is dependent on an intact host cell cytoskeleton and signaling pathways.} The invasion of epithelial cells by GCB-associated \textit{E. coli} induces alterations in the cell membrane characterized by elongations and actin condensation that are consistent with induced endocytosis by a “trigger” process (14). Internalized \textit{E. coli} appeared to be in a close vacuole, but its exact location within the endosomal lysosomal continuum remains to be determined.

\textbf{Salmonella enterica} serovar Typhimurium control was inhibited by cytochalasin D and genistein, but not colchicine or wortmannin.

\textbf{GCB-associated \textit{E. coli} persists in epithelial cells but not primary bone marrow-derived macrophages.} GCB strains KD1 to -3 and LF-82 were able to persist and replicate in epithelial cells over a 48-h period more effectively than strain KD4 and commensal DH5\textsubscript{A} (\(P < 0.05\)) (Fig. 8A).

In contrast to their behavior in epithelial cells, GCB-associated \textit{E. coli} and LF-82 were unable to survive longer than DH5\textsubscript{A} in cultured primary bone marrow-derived macrophages (\(P > 0.05\)) (Fig. 8B).

\textbf{GCB-associated \textit{E. coli} resembles Crohn’s disease-associated \textit{E. coli} LF 82 in phylogeny.} Serotyping revealed a different O:H serotype for each GCB isolate and LF-82 (Table 1). Evaluation of genotype by RAPD-PCR (Fig. 9) showed a unique banding pattern for each strain, indicative of diversity in overall genotype. Phylogenetic grouping of \textit{E. coli} strains by triplex PCR (Table 1) indicated that GCB strains KD-1 and KD-3 and CD strain LF-82 are part of the B2 phylogenetic group. The other two canine strains belonged to groups D and A. Phylogenetic triplex PCR performed on mucosal DNA samples from two affected Boxers gave a triple band pattern consistent with...
type B2. No bands were detected in the posttreatment mucosal DNA sample.

Multilocus sequence typing revealed that GCB strains KD1 to -3 and LF82 have novel st7 sequence types and clonal groups (Table 1). In contrast, GCB strain KD4 belonged to clonal group 23 that harbors predominantly E. coli isolated from healthy people (Selander strains) and a healthy dog (http://www.shigatox.net/cgi-bin/mlst7/strainquery?).

The genome sizes of LF-82 and KD1 to -3, strains that invade and persist within cultured cells, were between 188 and

FIG. 4. Fluorescence in situ hybridization of GCB mucosa with probes against Enterobacteriaceae and E. coli/Shigella. A. Multifocal clusters of bacteria (long arrow) that hybridize with probes E. coli 1531 (red; Enterobacteriaceae) and EUB-338 (green) are evident within the mucosa. A mixed population of Enterobacteriaceae (orange) and other bacteria (green) is visible on the luminal surface (small arrow). DAPI stained DNA (blue) (magnification, ×400). B. Selective mucosal invasion by clusters of bacteria (long arrow) that hybridize with a probe against E. coli/Shigella (red) but not non-EUB-338 (green). DAPI stained DNA (blue) (magnification, ×400). C. The mucosally invasive flora is composed of coccobacilli 1 to 3 μm long that hybridize with a Cy3-E. coli/Shigella-specific probe but not non-EUB-338 (green). DAPI stained DNA (blue) (magnification, ×600).

The results of statistical analysis are indicated by the letters A through D. Strains with different letters are significantly different from each other (P < 0.05). A. Adhesion to MAC-T epithelial cells. B. Invasion of MAC-T epithelial cells. C. Invasion of Caco-2 epithelial cells.

FIG. 5. Adhesion and invasion of cultured epithelial cells by E. coli associated with GCB (KD1 to -4) and Crohn’s disease (LF 82), commensal E. coli (DH5a), and Salmonella enterica serovar Typhimurium. The results of statistical analysis are indicated by the letters A through D. Strains with different letters are significantly different from each other (P < 0.05). A. Adhesion to MAC-T epithelial cells. B. Invasion of MAC-T epithelial cells. C. Invasion of Caco-2 epithelial cells.
FIG. 6. Microscopic examination of cultured epithelial cells infected with canine GCB-associated *E. coli* strain KD-2. A. Invasion of *E. coli* was confirmed by differential staining of intracellular and extracellular bacteria with differently labeled secondary antibodies before (FITC, green) and...
276 kbp larger than that of MG1655, whereas the genome size of KD-4 (4.37 Mbp), a strain that invades but does not persist, was similar to that of MG1655 (4.47 Mbp) (Table 1).

**GCB-associated** *E. coli* resembles Crohn’s disease-associated *E. coli* LF82 and extraintestinal pathogenic *E. coli* in virulence genes. PCR-based screening for virulence genes of diarrheagenic *E. coli* (LT, STa, STb, SLT-I, SLT-II, CNF-1, CNF-2, and the gamma variant of *eae*) and an invasion plasmid (*ipaH*) was negative for all GCB-associated strains (Table 1). All strains, including DH5α, were positive for *fimH*, the adhesin-encoding gene associated with epithelial cell invasion in uropathogenic *E. coli* (50). Sequencing to detect potentially pathoadaptive *fimH* alleles yielded polymorphisms at residues 27, 70, 78, 163, and 184 (Table 1). The translated sequences of the GCB isolate KD-1 and the Crohn’s disease isolate LF-82 were identical. Strains KD1, KD-3, and LF82 had the N70S or S78N mutation, reported to be lineage specific for the B2 phylogenetic group (31, 74).

A more comprehensive screening of virulence factors from diarrheagenic and extraintestinal pathogenic *E. coli* using microarray analysis showed that GCB-associated and LF 82 *E. coli* strains lacked invasion plasmids, the attaching and effacing *E. coli* type III secretion system-related genes, and toxins-encoding genes associated with virulence in intestinal or extraintestinal pathogenic *E. coli* (Table 2). GCB-associated and LF 82 strains shared a variety of genes implicated in iron acquisition and metabolism, notably *ipl1, ipl2, fyuA* (yersiniabactin), *chuA* (hemoglobin utilization), *fepC* (ferric enterobactin transport ATP-binding protein), and *iroN* (siderophore receptor), though aerobactin-related genes (*iutA* and *iucD*) were not detected. The *malX* gene, which is a marker for the pathogenicity island of UPEC CFT073, and the *iss* gene, encoding a protein responsible for serum resistance, were present in most strains. The overall virulence gene pattern was most similar for the GCB-associated isolates KD-1 and the CD isolate LF-82, and both of these strains contained the *ibeA* gene, encoding an invasin of meningitis-associated *E. coli* (3). Strain LF 82 also possessed the *usp* gene, which encodes a uropathogenesis-specific protein, but not any other UPEC-associated genes, such as *papA, papC, papGII* (pilus P-encoding genes), *cnf1* (cytotoxic necrotizing factor 1), or *afa* genes (afimbrial adhesin-encoding genes).

FIG. 7. Effects of inhibitors of microfilaments (cytochalasin D), microtubules (colchicine), tyrosine kinase (genistein), and PI 3-kinase (wortmannin) on invasion by IBD-associated *E. coli* and *Salmonella enterica* serovar Typhimurium. Invasion (percent of invasion in the absence of inhibitors) by GCB-associated *E. coli* (KD1 and KD2) was reduced by all inhibitors (*P* < 0.05). The results for LF 82 and the selective inhibition of *Salmonella* invasion by cytochalasin D and genistein but not colchicine or wortmannin are consistent with the literature. Results are expressed as means plus standard deviations.

FIG. 8. Survival of GCB-associated (KD1 to -4) and Crohn’s disease-associated (LF 82) *E. coli* in cultured epithelial cells and primary bone marrow-derived macrophages. A. Survival of *E. coli* over a 48-h period in cultured MAC-T epithelial cells, expressed as a percentage of the CFU recovered at 2 h. Strains KD1 to -3 and LF 82 replicate (**, *P* < 0.05), while DH5α and KD-4 decline. B. Survival of *E. coli* over a 72-h period in cultured primary bone marrow-derived macrophages. GCB- and CD-associated and commensal (DH5α) *E. coli* strains are not significantly different from each other and survive less well than *Salmonella* (*P* < 0.05).
DISCUSSION

This study directly explored the possibility that an uncharacterized infectious agent or an abnormal mucosal flora is involved in the etiopathogenesis of granulomatous colitis of Boxer dogs. Eubacterial FISH provided clear evidence that large numbers of coccobacilli are present within the colonic mucosa of Boxer dogs with granulomatous colitis, but not other types of canine colitis, and histologically normal tissues. Culture of the mucosas from two affected dogs yielded E. coli, Klebsiella, Streptococcus, Proteus, and Chryseobacterium, considered normal fecal flora in dogs (38). To create a more comprehensive inventory of the bacterial species inhabiting the colonic mucosa, including uncultivable bacteria, we generated 16S rDNA libraries from colonic biopsy samples of two GCB patients. These libraries were dominated by sequences for Enterobacteriaceae, predominantly E. coli and Shigella, concurred with the presence of intramucosal gram-negative cocccobacilli, and guided the selection of 16 and 23S rRNA FISH probes that hybridized with the invasive flora. The 16S E. coli/Shigella probe employed in the present study is specific for these species but cannot distinguish between them, so PCR for *ipaH*, a marker for the invasion plasmid of *Shigella* and enteroinvasive *E. coli*, and culture were performed to detect *Shigella*. As both PCR and culture were negative for *Shigella* we concluded that the invasive flora was *E. coli*.

Interestingly, *E. coli* was isolated previously from the regional lymph nodes of two Boxer dogs with granulomatous colitis at necropsy, but an association with disease was not determined (80). Another study, published while the present article was in preparation, describes the immunolocalization of *E. coli*, *Lawsonia intracellularis*, *Campylobacter*, and *Salmonella* to macrophages in the colons of 10, 3, 2, and 1 of 10 Boxer dogs with granulomatous colitis, respectively (79). While these findings lend independent support to our observations, their specificity is questionable because the antibody they used to localize *E. coli* (Dako B0357) is polyclonal, recognizes at least 80 different *E. coli* antigens in crossed immunoelectrophoresis and a multitude of *E. coli* antigens in immunoblotting from SDS-polyacrylamide gel electrophoresis, and is dilution dependent in its specificity (44, 81).

The association of *E. coli* with the intestinal mucosa of Boxer dogs with granulomatous colitis is similar to findings in people with inflammatory bowel disease, particularly Crohn’s disease, and rodents with engineered susceptibility to IBD (17, 49, 55, 72, 73, 76). However, the mucosal localization of *E. coli* in GCB, with multifocal clusters of *E. coli* consistently observed in the upper two-thirds of the mucosa, is more uniform than reported in human IBD, where some investigators describe intact bacteria, *E. coli* antigens, or DNA within granulomas and the lamina propria (11, 44, 55, 69) but others indicate that bacteria are restricted to the mucosal surface (40, 76). The variable localization of mucosa-associated *E. coli* in people

![FIG. 9. Genetic diversity of GCB- and CD-associated *E. coli* strains. RAPD-PCR of genomic DNA extracted from *E. coli* strains associated with Crohn’s disease (LF 82, lanes 1), and GCB (KD1 to -4, lanes 2 to 5) with RAPD primers 1254, 1281, and 1283.](image-url)

**TABLE 1. Characterization of GCB-associated *E. coli* strains**

| Strain | Serotype | Phylogroup | MLST (st7:cg) | Virulence
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>KD1</td>
<td>O8:H10</td>
<td>B2</td>
<td>N:N</td>
<td>—</td>
</tr>
<tr>
<td>KD2</td>
<td>O1:H+</td>
<td>D</td>
<td>N:N</td>
<td>—</td>
</tr>
<tr>
<td>KD3</td>
<td>O1:H4</td>
<td>B2</td>
<td>N:N</td>
<td>—</td>
</tr>
<tr>
<td>KD4</td>
<td>O145:H11</td>
<td>A</td>
<td>171:23</td>
<td>—</td>
</tr>
<tr>
<td>LF82</td>
<td>O83:H1</td>
<td>B2</td>
<td>N:N</td>
<td>ND</td>
</tr>
<tr>
<td>K-12 MG1655</td>
<td>OR:H48</td>
<td>A</td>
<td>173:0</td>
<td>ND</td>
</tr>
<tr>
<td>UPEC CFT073</td>
<td>06:H1</td>
<td>B2</td>
<td>27:38</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**a** MLST, multilocus sequence type; st7, sequence type based on seven housekeeping genes; cg, clonal group; ND, not determined in this study.

**b** PCR was performed for virulence genes, as follows: *E. coli* LT, STα, STβ, SLT-I, SLT-II, CNF-1, CNF-2, the gamma variant of *eae*, and an invasion plasmid (*ipaH*).

**c** AE014075.
TABLE 2. Microarray analysis for the detection of virulence genes from various diarrheagenic and extraintestinal pathogenic E. coli strains

<table>
<thead>
<tr>
<th>Gene*</th>
<th>Gene function</th>
<th>Microarray analysis result for strainb</th>
</tr>
</thead>
<tbody>
<tr>
<td>iss</td>
<td>Increased serum survival and surface exclusion protein</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>espMII</td>
<td>Protein involved in polysialic acid transport, group II</td>
<td>+ + + + -</td>
</tr>
<tr>
<td>ibeA</td>
<td>Protein of invasion</td>
<td>- + + - +</td>
</tr>
<tr>
<td>pic</td>
<td>Serine protease involved in colonization,</td>
<td>+ + - - -</td>
</tr>
<tr>
<td>malX</td>
<td>Malasse- and glucose-specific IAABC component,</td>
<td>+ + + + +</td>
</tr>
<tr>
<td></td>
<td>pathogenicity island associated</td>
<td></td>
</tr>
<tr>
<td>ipr1</td>
<td>Yersiniabactin biosynthetic protein</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>jpcE</td>
<td>Ferric enterobactin transport ATP-binding protein</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>bfpA (O1113)</td>
<td>Major subunit of long polar fimbriae LPF (variant O1113)</td>
<td>- - - + -</td>
</tr>
<tr>
<td>fimbA4</td>
<td>Flagellin (fliC variant)</td>
<td>- - - - +</td>
</tr>
<tr>
<td>troN</td>
<td>Siderophore receptor</td>
<td>+ + + - -</td>
</tr>
<tr>
<td>focA</td>
<td>Major fimbrial subunit of FIC fimbrae</td>
<td>+ + - - -</td>
</tr>
<tr>
<td>lpfA</td>
<td>Major subunit of long polar fimbriae LPF</td>
<td>- + - - -</td>
</tr>
<tr>
<td>ipr2</td>
<td>Yersiniabactin biosynthetic protein (putative ligase)</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>fyuA</td>
<td>Pesticin/yersiniabactin receptor protein</td>
<td>+ + + - +</td>
</tr>
<tr>
<td>colY</td>
<td>Colicin Y structural protein</td>
<td>- - - - +</td>
</tr>
<tr>
<td>mchB</td>
<td>Microcin H47 structural protein</td>
<td>+ + - - -</td>
</tr>
<tr>
<td>ccdB</td>
<td>Cytotoxic protein</td>
<td>- - - - +</td>
</tr>
<tr>
<td>cia</td>
<td>Colicin Ia structural protein</td>
<td>- - - - -</td>
</tr>
<tr>
<td>traT</td>
<td>Complement resistance protein</td>
<td>- - - + +</td>
</tr>
<tr>
<td>rtx</td>
<td>Putative RTX family exoprotein</td>
<td>- - - + -</td>
</tr>
<tr>
<td>chuA</td>
<td>Outer membrane receptor protein, heme utilization/transport protein</td>
<td>+ + + + -</td>
</tr>
<tr>
<td>usp</td>
<td>Uropathogenesis-specific protein</td>
<td>- + + + +</td>
</tr>
</tbody>
</table>

* For all information about the oligonucleotide probes and the genes targeted by the DNA microarray, see reference 7a.

+ and - indicate positive and negative microarray hybridization results, respectively. Oligonucleotide probes which gave positive results for at least one of the tested strains and also for the reference strain K-12 are not shown in this table. For all strains, positive results were obtained with ompT- and fimH-specific probes.

with IBD may be attributable to differences in disease phenotyping between studies (15), biopsy site (ileal, colonic, or rectal), biopsy type (surgical versus endoscopic) (52), and method used, e.g., FISH (the 23S E. coli 1551 probe employed by previous studies [55, 72] is not E. coli specific). It could also reflect the presence of E. coli strains that differ in their ability to invade and persist within the mucosa. The latter possibility is supported by observations that strains with an ability to adhere to and invade cultured epithelial cells, hallmarks of pathogenic E. coli, are more commonly isolated from patients with Crohn’s disease than those with ulcerative colitis or healthy controls (17, 49): i.e., such strains are found in 36% of early CD lesions in the ileum, compared with 3.7% of colonic CD, 0% of UC, and 1.9% of control samples. In the present study we found that GCB-associated E. coli was able effectively to adhere to and invade cultured epithelial cells in numbers similar to those of the well-characterized ileal CD-associated E. coli strain, LF-82 (16). GCB E. coli, like LF-82, was also able to invade a diverse spectrum of cultured epithelial cell lines (5). Transmission electron microscopy of canine GCB E. coli, and PI 3-kinase, and is consistent with previous studies with LF-82 in Hep2 and Int 407 epithelial cells (5). Microtubules and microfilaments are also utilized by enteroinvasive and meningitis-associated E. coli and invasive Klebsiella and Campylobacter but not Salmonella (22, 53, 57, 61).

Invasive GCB-associated E. coli was able to persist in cultured epithelial cells for 48 h and appeared to reside in a tight vacuole within the cytoplasm, suggesting a location in the endosomal lysosomal continuum. E. coli LF 82 has also been shown to reside and replicate in the cytoplasm of epithelial cells (5). Intracellular persistence and replication suggest an ability to escape from the endosomal and lysosomal network and parallel the situation with the pathogens Shigella and Listeria (14). Intracellular persistence of IBD-associated E. coli may directly contribute to the proinflammatory mucosal environment in IBD, and this is supported by studies demonstrating translocation of NF-κB and release of IL-8 in epithelial cells infected with IBD-associated E. coli (27, 42).

Our findings that GCB E. coli isolates and LF 82 did not outlive commensal E. coli (DH5α) in primary bone marrow-derived macrophages contrast with previous reports describing survival and replication of LF 82 relative to harmless commensal E. coli in cultured J774-A1 macrophages and a survival advantage in human-derived mononuclear macrophages and mouse peritoneal cells (7, 27). Those observations appear contradictory, but they can be reconciled by considering the relatively weak killing ability of the J774-A1 cell line relative to that of primary macrophages (27). In the light of recent studies showing impaired innate immunity in the form of an abnormal acute inflammatory response to E. coli in people with Crohn’s disease (47), different outcomes based on phagocytic ability
may be analogous to differences between healthy and susceptible individuals, with host susceptibility (e.g., predisposition of Boxer breed for GCB, polymorphisms in NOD-2) and disease-associated luminal bacteria (e.g., IBD-associated *E. coli*) acting as joint determinants of disease.

The combination of phylogenetic analysis and virulence gene profiling provided insights into the lineage and genetic armory of GCB and Cohn’s associated *E. coli*. The presence of *chuA*, determined by PCR and microarray analysis, placed three of four GCB strains and LF 82 into group B2 or D. The presence of *yjaA* indicated that *E. coli* KD-1, KD-3 and LF 82 are B2, while its absence placed KD-2 into group D (12). The detection of the *fumH* polymorphisms associated with B2 lineage, N70S and S78N, further supported these results (31, 74). The serotypes (O1, O8, and O83) and gene profiles (*fyuA, irp1, irp2, malX, ompT, ibeA* and *kpsMII*) of these B2 and D strains are consistent with those of extraintestinal pathogenic *E. coli* (ExPEC), which causes cystitis, pyelonephritis, prostatitis, sepsis, and meningitis, and avian pathogenic *E. coli* (*E. coli* (4, 21, 26, 36, 37). In contrast, strain KD-4, our least invasive and persistent GCB isolate, belonged to group A, which contains most of the commensal strains of *E. coli* (62). The mucosal association of B2 *E. coli* in GCB was confirmed by phylogenetic triple PCR of colonic DNA that yielded amplicons for *chuA, yjaA*, and TSPE4.C2 in samples from two GCB-affected dogs but not the postremission sample. These observations suggest that GCB- and CD-associated *E. coli* strains are genetically more similar to ExPEC than diarrheagenic *E. coli* strains.

Precise placement of GCB-associated *E. coli* strains and LF 82 within the ExPEC group is difficult. For example, the gene profiles of KD1 to -3 and LF 82 are broadly similar to that of UPEC CFT073: all contain *malX*, a marker of a pathogenicity island in CFT073, and strain LF 82 also hybridizes with a uropathogenesis-specific gene (*uag*), but these strains lack genes, such as *papA*, *papC*, *papGII*, *cnf1*, *afa*, *hlyA*, and *ucuD*, that are commonly associated with UPEC and other ExPEC strains (3, 21, 35, 36). The presence of *ibeA*, the gene encoding invasin for brain endothelium, in KD-1 and LF 82 suggests these strains may belong to the meningitis-associated group (3). However, *ibeA* is not restricted to meningitis-causing strains, and KD-1 and LF 82 lack genes, such as *sfa*, *cdtB*, *neuA*, and *neuC*, that are present in many B2 meningitis strains (26, 36). Moreover, GCB-associated *E. coli* and LF 82 are able to invade and persist in epithelial cells, whereas invasion by meningitis-associated *E. coli* is restricted to endothelial cells (53).

The pathogen-like behavior displayed by GCB and CD *E. coli* isolates in cultured cells strongly suggests that these strains harbor genes encoding virulence, but an extensive PCR- and microarray-based screen of GCB-associated and LF 82 *E. coli* strains failed to detect genes involved with the pathogenic behavior of *E. coli* strains associated with intestinal disease, such as invasion plasmds, type III secretion systems, or toxins, and concurs with previous studies of CD-associated *E. coli* in people (16, 17, 42, 49). The few virulence genes we found in GCB-associated *E. coli* and LF 82 were largely part of a cluster of genes, *irp1, irp2, fyuA* (yersiniabactin), *chuA* (hemoglobin utilization), *fepC* (terric enterobactin transport ATP-binding protein), and *iroN* (siderophore receptor), involved in iron acquisition and metabolism (13, 41). These genes are considered important for iron acquisition by ExPEC within an infected host, and bacterial siderophores may also impact the cellular immune response (23).

The paucity of virulence genes detected in GCB and CD *E. coli* strains suggests that these strains may harbor as-yet-uncharacterized genes to account for their disease association and pathogenic behavior in cultured cells. This seems feasible considering the high degree of diversity in *E. coli* as a species (only 39% of core proteins are conserved in UPEC, EHEC, and K-12) and its propensity for acquiring DNA from distantly related organisms (56, 83). The B2 lineage is also a particularly appropriate genetic background for acquiring virulence traits: B2 strains are associated with lethality in mice (21, 31, 62) and often have larger genome sizes than the commensals in group A, reflecting the presence of virulence-associated genes such as pathogenicity islands (29, 35, 62). Our findings that GCB strains KD1 to -3 and LF 82 have larger genomes than *E. coli* MG1655, belong to undefined MLST clonal groups, and contain genes that are thought to be acquired by horizontal transfer (e.g., the *yersiniabactin* gene and *malX*) (71) further support this possibility and the notion that this group of *E. coli* strains represents a new pathotype, adherent and invasive *E. coli* (AIEC), as proposed by Darfeuille-Michaud et al. (16). Strains with an AIEC pathotype could potentially belong to a clonal group associated with chronic intestinal inflammation, comparable to the association of O157:H7 with hemorrhagic gastroenteritis and hemolytic uremic syndrome. However, serotyping and genotyping (with random amplified polymorphic DNA PCR) showed marked heterogeneity between strains and does not support the presence of a unique *E. coli* strain associated with GCB and Crohn’s disease. These observations are similar to the results of ribotype analysis of *E. coli* strains from patients with CD showing that no single strain is found in every patient (51).

In conclusion, we have determined that granulomatous colitis of Boxer dogs, a disease that has features in common with idiopathic IBD in people, is associated with selective intramuscosal colonization by *Escherichia coli*. *E. coli* strains isolated from the mucosas of two affected dogs adhere to, invade, persist in, and replicate in cultured epithelial cells to the same degree as Crohn’s disease-associated *E. coli* LF-82. The invasion process, which resembles triggered endocytosis, requires intact microtubules, microfilaments, PI 3-kinase, and tyrosine kinase. The similar phylogeny and virulence gene profiles of GCB strains and LF 82 hint at the possibility of lineage-specific pathoadaptation and point to the association of *E. coli* strains resembling extraintestinal pathogenic strains in genotype with chronic intestinal inflammation in dogs and people. The role of these *E. coli* strains in the etiopathogenesis of GCB and CD remains to be determined.

** ACKNOWLEDGMENTS**

K. Simpson is supported by a grant from the U.S. Public Health Service (DK002938). This study was funded in part by the Indirect Vitamins Purchasers Antitrust Litigation Settlement administered by the New York State Attorney General.

We thank J. Chatman, D. J. Chew, M. J. Day, E. J. Hall, R. A. Hostutler, and D. F. Kelly for providing patient information and tissue sections, A. Darfeuille-Michaud and T. S. Whittam for *E. coli* strains, and A. Quaroni for the Caco-2 cell line. We thank Francis Davis for technical support.